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13. ABSTRACT (Maximum 200 Words)

We have made considerable progress towards testing the proposed hypothesis that the signaling proteins Rac3 and PAK are critical for the initiation of breast cancer metastasis. Stable fluorescent protein-tagged breast cancer cell lines expressing active and inactive forms of Rac3: MDA-MB-435 highly metastatic breast cancer cell line expressing dominant negative Rac3 and Hs578t non-metastatic breast cancer cell line expressing dominant active Rac3 have been created. We have analyzed these cell lines *in vitro* prior to *in vivo* analysis in a nude mouse model. As expected, metastatic cell lines expressing dominant active Rac3 demonstrate reduced migratory and adhesive properties. The non-metastatic breast cancer cell lines expressing dominant negative Rac3 demonstrate increased migratory and adhesive properties. We have initiated *in vivo* studies by injection of non metastatic breast cancer cells expressing Rac3(T17N) and the control cells into the mammary fat pads of female nude mice. We are currently monitoring for changes in metastatic efficiency. To provide a direct assessment of the functional consequences of activating or inhibiting Rac3, we have initiated the adaptation of a fluorescence confocal microscope to specifically image fluorescence cells inside live mouse tumors. We expect to first phase of our image analysis to begin in May 2003.

14. SUBJECT TERMS
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Introduction

Cancer metastasis is a complex multistep process in which malignant cells escape from a primary tumor, invade surrounding tissue, migrate through the extracellular matrix, and are transported via the circulatory system to establish secondary tumors at distant sites (1-3). Rho family of small GTPases are key regulatory molecules that have been implicated in cell invasion (4; 5). The small GTPase Rac3 is a closely related homologue of the Rho GTPases Rac1 and Cdc42, which have been shown to regulate actin cytoskeletal reorganization during cell invasion (6). Rac3 is constitutively active in aggressively dividing, high metatsatic breast cancer cell lines and tissues. Transient expression of dominant active Rac3, activated DNA synthesis and conferred a highly proliferative phenotype to human mammary epithelial cells via activation of a downsteam effecctor, P21-activated kinase (PAK) (7). PAKs are a group of 62-68 kDa serine/threonine kinases that have been identified as targets of activated Rac and Cdc42 (8-11). PAKs have been implicated in the regulation of the stress-activated MAP kinases, p38 and JNK, cytoskeletal rearrangement, cell-extracellular matrix interactions, cell motility, and apoptosis (9; 12-14). In vitro studies using breast cancer cell lines have shown that PAK regulates anchorage-independent growth, mitotic spindle organization, tumorigenicity, and angiogenesis as well as cytoskeletal reorganization, cell migration, and invasion (15-19). These are all cell functions that are expected to be dysregulated in metastatic cancer. However, a role for PAK and Rac3 as metastasis promoters *in vivo* has yet to be substantiated.

Hypothesis

Therefore, we hypothesized that the signaling proteins Rac3 and PAK are critical for the initiation of metastasis. To test our hypothesis we have created breast cancer cell lines expressing mutant forms of Rac3 that have been analyzed *in vitro* and are now beginning to be analyzed *in vivo*.

Results
Vector construction

Wild type and Rac3 cDNA containing aminoacid substitutions that render the expressed protein dominant negative (T17N), dominant active (G12V), or highly active (F28L) were obtained from Dr. Ulla Knaus, Department of Immunology, Scripps Research Institute, La Jolla, CA. These constructs were successfully cloned into bicistronic vectors that express red fluorescent protein (RFP) and neomycin resistance (Clontech). These constructs have also being cloned into a tetracycline responsive retroviral vector (Rev.TRE, Clontech) and we are currently in the process of making cell lines that express the TtA protein, which would transactivate the inducible promoters on the Rev. TRE. Rac3 vectors.

Cell Lines

Stable breast cancer cell lines expressing the bicistronic vectors containing active and inactive forms of Rac3 have been created by Lipofectamine transfection followed by selection on neomycin.

The following cell lines have been created:
1. MDA-MB-435 highly metastatic breast cancer cell line expressing dominant negative form of Rac3, Rac3(T17N).
2. Hs578t non-metastatic breast cancer cell line expressing active fast cycling form of Rac3, Rac3(F28L).

3. A low metastatic variant of the MB-435 cell line (MB-435 Br) expressing Rac3(F28L), a highly active form of Rac3.
4. MB-435 Br breast cancer cell line expressing dominant active Rac3, Rac3(G12V).

Characterization of mutant breast cancer cell lines

1. *Characterization of MB-435 (Rac3T17N)*
A. Western analysis for expression levels
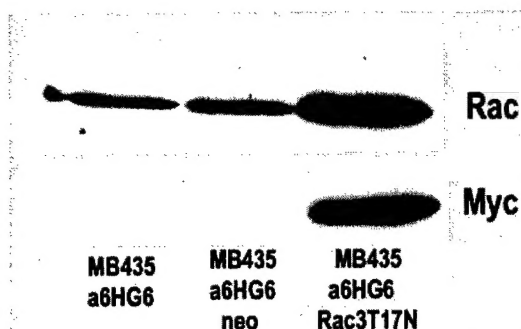
Figure 1. Western analysis of MB-435 high metastatic breast cancer cell lines expressing Rac3(T17N). Equal total protein amounts of whole cell lysates of MB 435 cells (left lane), MB-435 cells expressing the neomycin resistant bicistronic vector (middle lane), or MB-435 cells expressing Rac3(T17N) were western blotted with either anti Rac3 (top row) or anti myc (bottom row).

Since a previous report demonstrated that Rac3 was activated specifically in the more metatstaic malignant breast cancer cell line MDA-MB-435, we stably expressed dominant negative Rac3(T17N) in a highly metastatic variant of this cell line. Figure 1 demonstrates a typical western from a clone expressing a myc-tagged Rac3 mutant protein. Proteins from total cell lysates were western blotted for Rac using a pan Rac antibody (Santa Cruz Biotech., CA) or an anti myc antibody (Sigma, MO). Immunoblots were detected with the SuperSignal West Femto-Substrate chemiluminescence kit (Pierce Endogen, IL) and Kodak Biomax MR film (Fisher Scientific, TX). Typical Rac3(T17N) clones demonstrated elevated mutant Rac3 protein expression by about 5-10 times more than wild type (Fig. 1).

B. In vitro mutant analysis
Cells expressing Rac3(T17N) were analyzed for alterations in their motile and invasive potential in response to extracellular matrix components. Modified Boyden chambers (tissue culture treated, 6.5 mm diameter, 10μm thickness, 8 μm pores, Transwell®, Costar Corp., Cambridge, MA) were coated on the underside (haptotactic migration), or the upper surface (invasion), of the membrane with matrigel (Fisher Scientific, TX), 50 μg/ml laminin, or 50 μg/ml fibronectin (Gibco BRL, MD) overnight at 4° and then placed into the lower chamber containing 500 μl culture media with 10% fetal bovine serum (FBS). Serum starved cells (10^5 cells) were added to the upper surface of each migration chamber and allowed to migrate to the underside of the membrane for 4 hours (migration) or 24 hours (invasion). The non-migratory cells on the upper membrane surface were removed with a cotton swab, the migratory cells attached to the bottom surface of the membrane stained with propidium iodide (CalBioChem-Novabiochem Corp., CA) and quantified.

Expression of Rac3(T17N) in a highly metastatic variant of the MB-435 breast cancer cell line (MB435.α6HG6) resulted in a statistically significant decrease in invasion through laminin,

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matrigel, and fibronectin. Haptotatic migration towards laminin was also significantly decr eased in the MB435.α6HG6 cells expressing Rac3(T17N) (Fig.2).
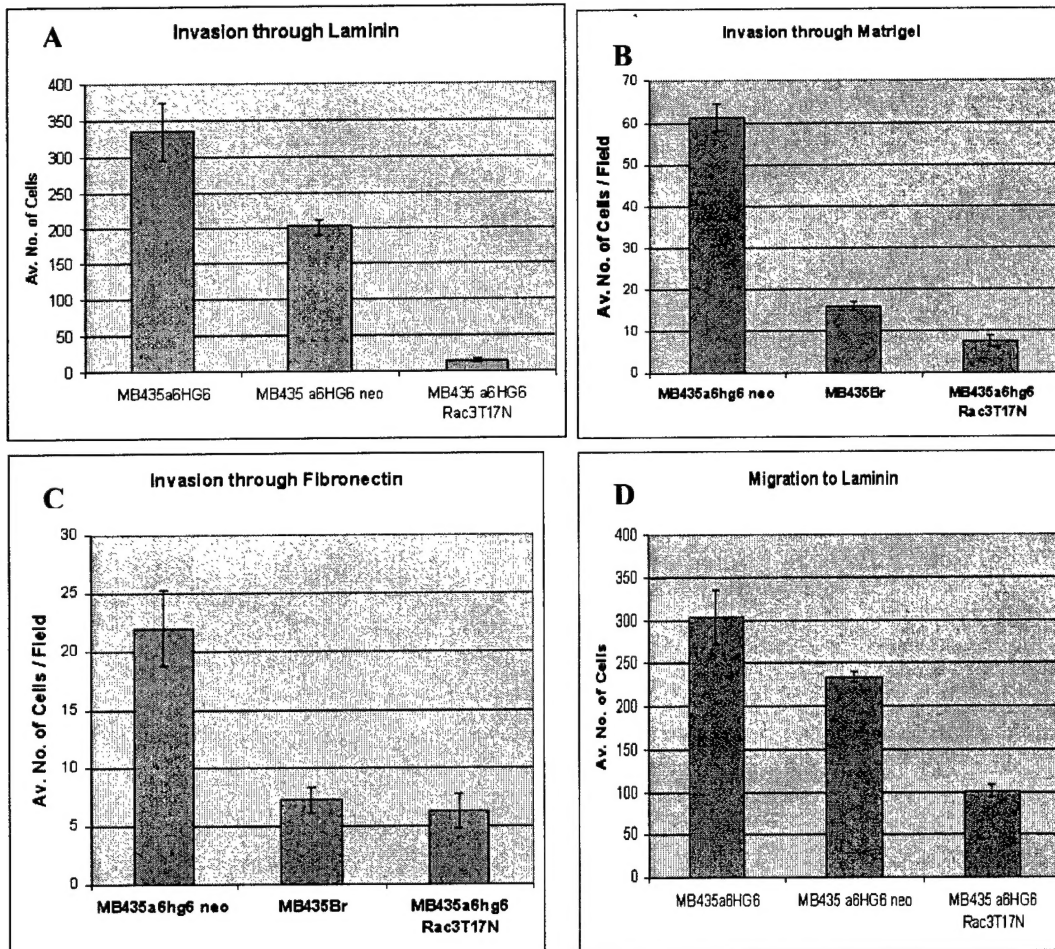
Figure 2. Invasion and migration assays of control and Rac3(T17N) expressing MB-435 cells. Quiescent MB435.α6HG6 (high metatstatic), MB435.α6HG6neo (vector control), MB435.α6HG6 Rac3(T17N), or MB-435 Br (low metatstaic) breast cancer cell lines were plated onto the membrane of a Transwell (Costar, MA). Invasion assays across laminin **(A)**, matrigel **(B)** or fibronectin **(C)**. The cells that invaded to the underside of a membrane coated on top with ECM proteins were stained with PI and counted at (x400). Y-axis represents the number of cells/microscopic field for at least 10 microscopic fileds/cell line.

Migration assay across laminin **(D)** was conducted by plating quiescent cell strains onto the membrane of a Transwell (Costar, MA) coated on the underside with laminin. The cells that migrated to the underside of the membrane were stained with PI and counted at (x400).

Y-axis represents the number of cells/microscopic field for at least 10 microscopic fields/cell line. Error bars represent +/- SEM.
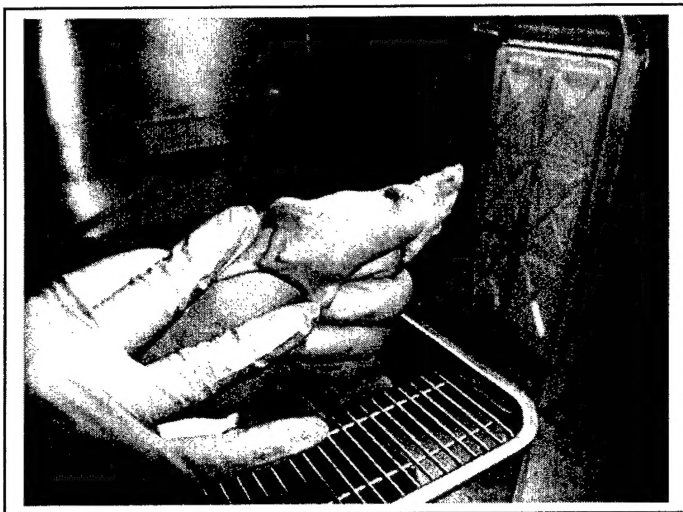
Figure 3. Primary tumor with MB-435 high metastatic breast cancer cells expressing Rac3(T17N). Approximately 0.1 ml of about $1X10^6$ MB-435 (Rac3T17N) cells were injected into the mammary fat pad of anesthetized female nude mouse and examined daily for tumor formation.

C. In vivo mutant analysis

We have initiated an *in vivo* analysis of MB435.α6HG6 cells expressing Rac3(T17N) to investigate whether dominant negative Rac3 expression affects metastatic efficiency in the nude mouse model. Contrrol and MB-435 Rac3(T17N) cells were injected into the mammary fat pad of female nude mice. Two weeks following inoculation, both control and Rac3(T17N) expressing mice demonstrated similar size tumors (Fig. 3). We are currently analyzing these mice for potential differences in metatstaic potential.
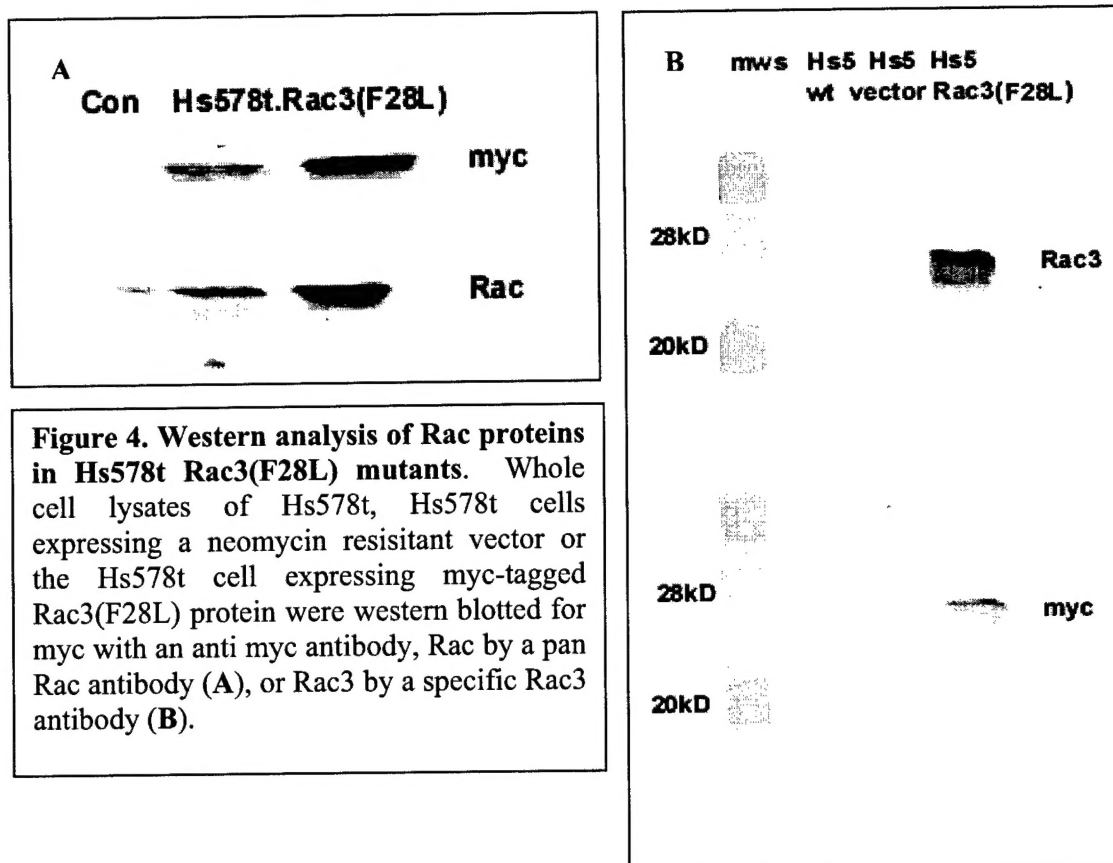
2. *Characterization of Hs578t (Rac3F28L)*

A. Western analysis for expression levels

Since previous data demonstrated decreased Rac3 expression in the Hs578t non-metastatic human breast cancer cell line when compared to the high metatstaic MB-435 cells (7), a fast cycling active form of Rac3, Rac3(F28L) was stably expressed in Hs578t cells. Myc-tagged Rac3(F28L) expressing Hs578t cells were isolated by neomycin resistance. Whole cell lysates were western blotted using a pan Rac antibody (Santa cruz Biotech., CA), a Rac3 antibody (kind gift of Dr. Ulla Knaus) or an anti myc antibody (Sigma, MO). Control Hs578t cells expressing vector alone, and Hs578t Rac3(T17N) cells expressed Rac proteins as detected by a pan Rac antibody (Fig. 4A). This antibody detects both Rac1 and 3 as a 21 kD band. The Rac3 specific antibody only detected Rac3 in the Rac3(F28L) expressing Hs578t cells, thus confirming that the parent Hs578t cell line expressed little or no Rac3 (Fig. 4B).

The altered activity of mutant Rac3 proteins in the stably transfected cell lines was also confirmed by analysis of Rac3 activity using the i*n vivo* assay described in (7; 20) (Fig. 5). This technique is based on the fact that the Cdc42 and Rac interacting domain (CRIB) of Rac downstream effectors binds specifically to the GTP bound active form of Rac. A fragment encoding the CRIB domain from PAK was used to yield A GST-PAK-CRIB fusion protein. Cell lysates incubated with GST-PAK-CRIB fusion protein bound to glutathione coupled sepharose beads were eluted in Laemmli sample buffer and western blotted for Rac3 using specific antibodies. As expected, the Hs578t cells expressing Rac3(F28L) demonstrated high activity both in the presence and absence of GTP (Fig. 5). The control cells did not demonstrate any Rac3 activity (data not shown).

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A

Con   Hs578t.Rac3(F28L)

                                    myc

                                    Rac

B   mws Hs5 Hs5 Hs5
         wt vector Rac3(F28L)

28kD

20kD   Rac3

28kD   myc

20kD

Figure 4. Western analysis of Rac proteins in Hs578t Rac3(F28L) mutants. Whole cell lysates of Hs578t, Hs578t cells expressing a neomycin resisitant vector or the Hs578t cell expressing myc-tagged Rac3(F28L) protein were western blotted for myc with an anti myc antibody, Rac by a pan Rac antibody (**A**), or Rac3 by a specific Rac3 antibody (**B**).

Lys -GTP,S -GTP,P +GTP,S +GTP,P
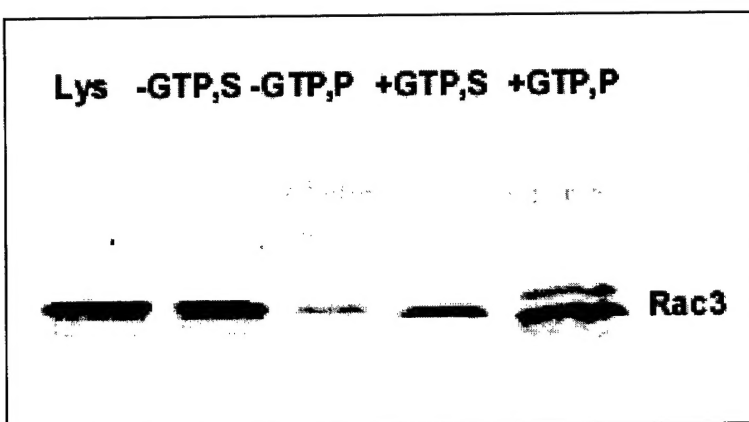
                                    Rac3

Figure 5. Rac3 activity in Hs578t Rac3(F28L) breast cancer cells. Rac3 activity in whole cell lysates of Hs578t Rac3(F28L) cells was determined by a pull down assay using a GST-PAK-CRIB fusion protein. Equal loading was maintained by performing a total protein assay. **Lys,** total Rac3; **–GTP,S,** Supernatant in the absence of added GTP; **-GTP,P,** pellet in the absence of added GTP; **+ GTP, S,** supernatant in the presence of GTP; **+GTP,P,** pellet in the presence of GTP.

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B. In vitro mutant analysis
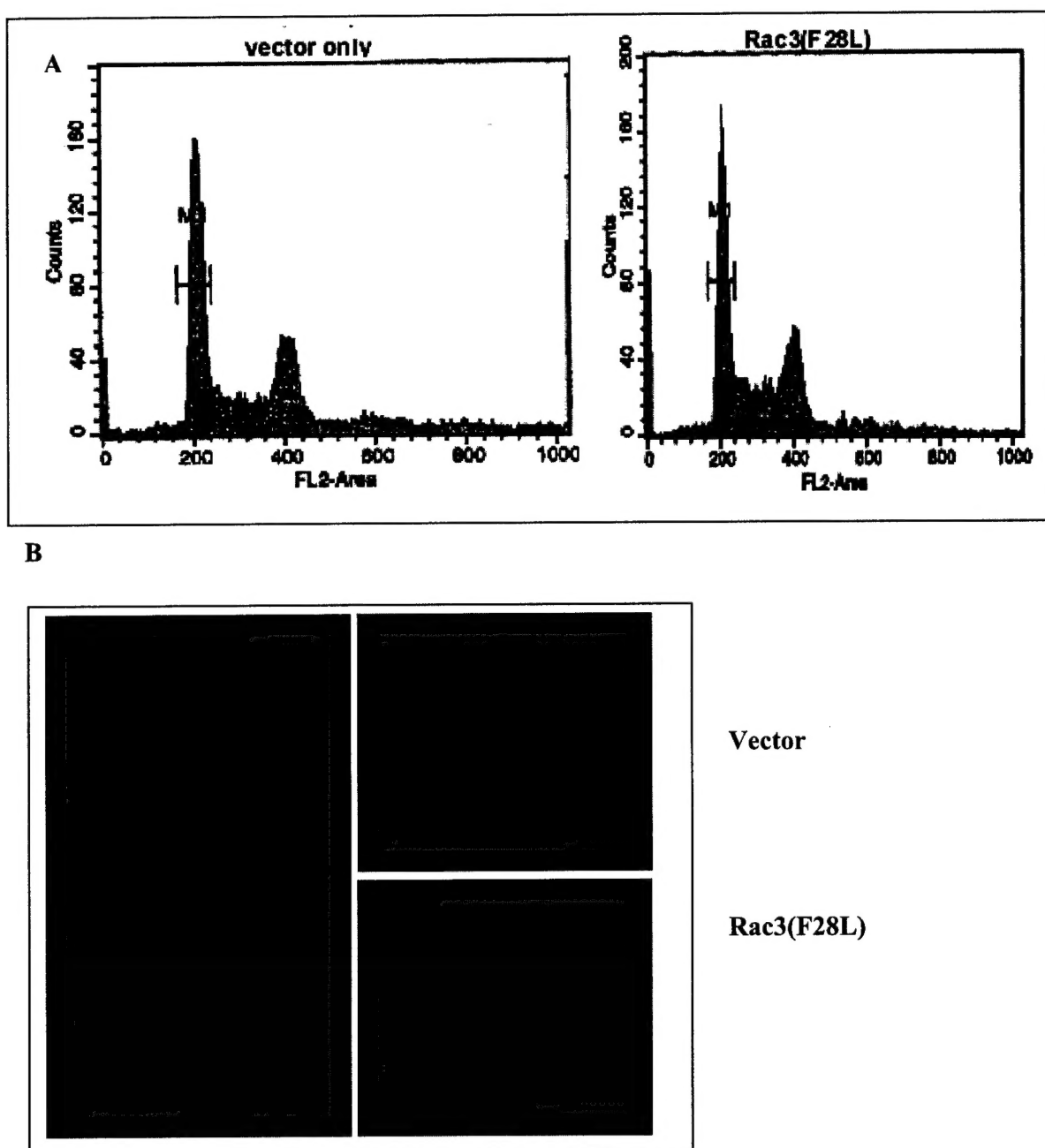
Figure 6. Characterization of Hs578t Rac3(F28L) cells.
A. FACS analysis of Hs578t cells expressing vector alone or Rac3(F28L) amino acid substitution.
B. Fluorescence microscopy of Hs578t cells expressing Rac3 mutants stained with Rhodamine phalloidin for F-actin.

Fluorescence activated cell sorter (FACS) analysis was conducted on propidium iodide stained Hs578t control and Rac3(F28L) expressing cell lines to determine the potential effects of expressing Rac3(F28L) on cell cycle progression in Hs578t cells. The expression of Rac3(F28L) did not significantly alter cell cycle stage when compared to control cells (Fig. 6A).

To determine whther Rac3(F28L) expression affected the motile phenotype of Hs578t cells, cells were fixed and stained for F-actin using Rhodamine phalloidin, as described previously (21). Cells expressing Rac3(F28L) demonstrated a dramatic increase in organization of polymerized actin at the leading edge to form lamellipodia and membrane ruffles, structures associated with Rac-regulated cell motility. We are currently analyzing these cell lines for increased migration in Boyden chamber assasys.

C. *In vivo mutant analysis*
The Hs578t parental cell line did not form significant tumors in the mammary fat pads of nude mice. We are currently attempting to produce tumors in the nude mouse model using Hs578t Rac3(F28L) cell line.

3. *Characterization of MB-435Br cells expressing dominant active Rac3*

To ensure successful establishment of primary mammary tumors, we have taken an alternate approach by creating Rac3(F28L) and Rac3(G12V) dominant active mutations in a low metastatic variant of MB-435 cells (MB-435Br) that forms primary tumors in nude mice. We are currently in the process of analyzing these cells both *in vitro* and *in vivo*. As expected, cells expressing dominant active forms of Rac3 demonstrate a more motile phenotype with more membrane ruffles (Fig. 7).
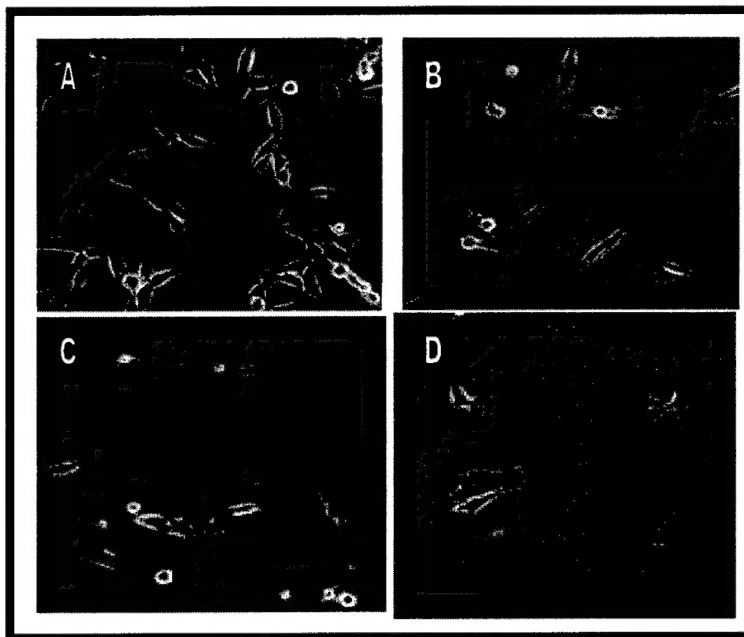

Figure 7. Phenotype of MB435Br low metastatic cells expressing Rac3 mutants. A, Control cells; B, Vector only; C, Rac3(F28L), D, Rac3(G12V) expressing cells.

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In vivo imaging

The overall focus of this proposal is to develop and establish direct *in vivo* imaging for the functional analysis of signaling proteins involved in breast cancer metastasis. To this end we are adapting a confocal fluorescence microscope to image live animals with fluorescent breast tumors. Currently this microscope is under construction and we are testing fluorescent filters.

Prior to image analysis in the confocal microscope, which requires anesthesia and possible surgery to create a skin flap, we feel that the mice will be screened using a simple light box as indicated in the following diagram (Fig. 8). This system consists of a light source, filtered to provide optimal tissue illumination; an imaging device; and a filter used to create optimal observation conditions.
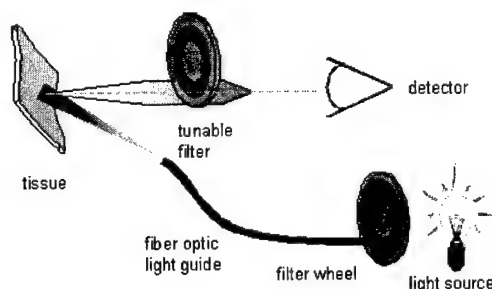

Figure 8. Schematic diagram of the Vision Enhancement System concept.

The filter wheel can be set to transmit white light for tissue reflectance evaluation, or to provide optimally selected excitation illumination for fluorescent cells.
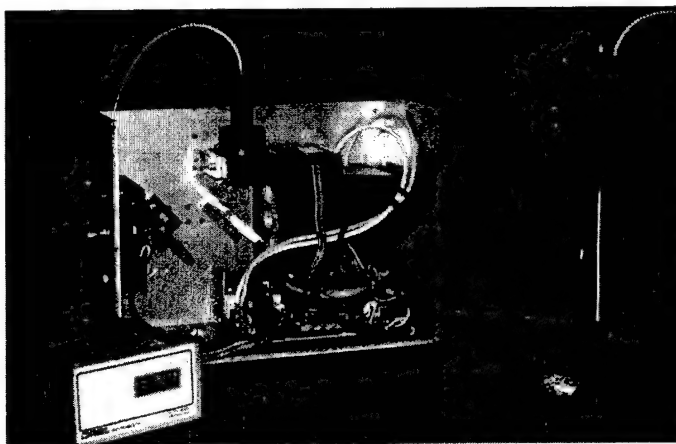
Figure 9. Vision Enhancement System used to image autofluorescence from live mice. The Xenon source is filtered with bandpass filters placed in a filter wheel regulated with a control box; light is directed to image with a fiberoptic cable. Observation filters are placed in front of 5mm camera.

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A light source was constructed to provide optimal illumination according to the calculated ideal conditions. Creation of this vision enhancement system is expected to enable us to image the nude mice carrying fluorescent tumors, prior to more in-depth analysis in the confocal fluorescent microscope. Fig. 10 demonstrates a plate of RFP-expressing Hs578t breast cancer cells under this system. This clearly illustrates the utility of this system to image gross morphology of tumors created with FP-expressing wild type and mutant Rac3 cell lines.
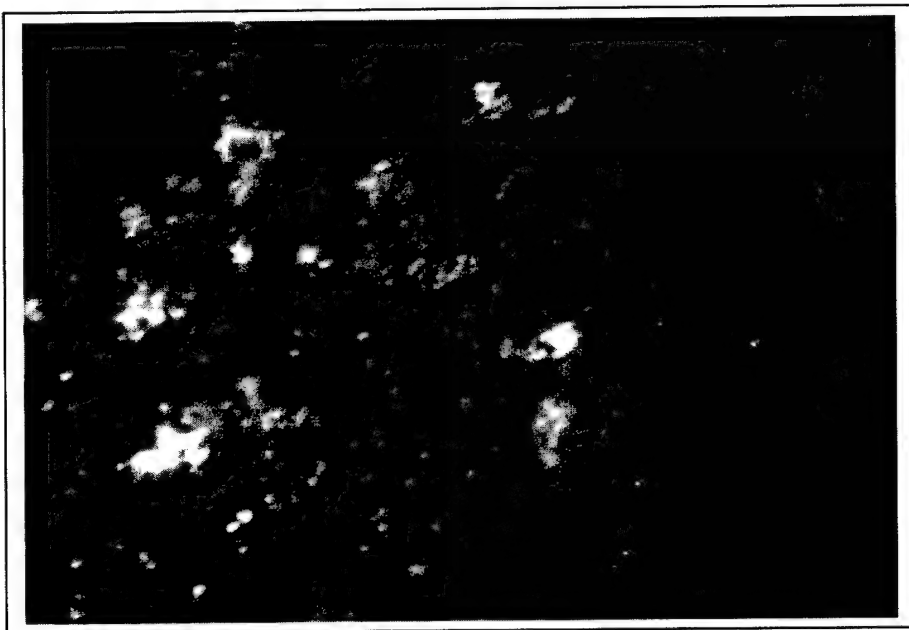
Figure 10. RFP-tagged Hs578t cells on Petri dish as viewed by vision enhancement system under red fluorescent filter.

Key Research Accomplishments

Stable cell line created	*In vitro* mutant analysis	*In vivo* mutant analysis
1. MDA-MB-435 (high metastatic breast cancer cell line) expressing dominant negative form of Rac3, Rac3(T17N).	Western analysis, Rac3 activity assays, invasion, and migration assays completed.	Currently under analysis.
2. Hs578t (non-metastatic breast cancer cell line) expressing active fast cycling form of Rac3, Rac3(F28L).	Western analysis, Rac3 activity assays, analysis of cell cycle stage, phenotype of actin cytoskeleton completed.	Initiated.
3. A low metastatic variant of the MB-435 cell line (MB-435 Br) expressing Rac3(F28L), a highly active form of Rac3.	Western analysis and phenotype of actin cytoskeleton completed.	To be conducted.
4. A low metastatic variant of the MB-435 cell line (MB-435 Br) expressing dominant active Rac3(G12V).	Western analysis and phenotype of actin cytoskeleton completed.	To be conducted

Reportable Outcomes

Our *in vitro* analysis of the consequence of expressing a dominant negative Rac3 (Rac3.T17N) in highly metastatic variant of MB-435 human breast cancer cell line is complete. We expect the *in vivo* histopathological analysis of distant metastases to be completed at the end of the month. Therefore, this work is currently in preparation for submission to the journal, Cancer Research; "Krisnhamoorty, L., Baugher, P., Knaus, U., Price, J., and Dharmawardhane, S. Stable expression of dominant negative Rac3 reduces the metastatic efficiency of a highly metastatic variant of MDA-MB-435".

Conclusions

The experiments proposed for the first year in the statement of work has been accomplished.

As proposed in our task 1 for the first 18 months;,

A. Expression vectors containing the following mutations Rac3 and PAK have been created:

    I.   Rac3(F28L) : dominant active fast cycler

    II.   Rac3(G12V): dominant active

    III.   Rac3(T17N): dominant negative

    IV.   PAK1(83-149): autoinhibitory domain (AID)

    V.   PAK1(83-149, L107F): ineffective AID

B. Stable human breast cancer cell lines containing the following mutations have been constructed:

    I.   MDA-MB-435 highly metastatic breast cancer cell line expressing dominant negative form of Rac3, Rac3(T17N).

    II.   Hs578t non-metastatic breast cancer cell line expressing active fast cycling form of Rac3, Rac3(F28L).

    III.   A low metastatic variant of the MB-435 cell line (MB-435 Br) expressing Rac3(F28L), a highly active form of Rac3.

    IV.   MB-435 Br breast cancer cell line expressing dominant active Rac3, Rac3(G12V).

C. We have initiated the proposed *in vitro* characterization of the transfected cells expressing mutant Rac3.

    I.   Western blotting of cell lysates with anti myc and anti Rac antibodies.

    II.   Assays to determine activation status of mutant Rac3 proteins using the specific active Rho GTPase binding domain of PAK.

    III.   Fluorescence microscopy to investigate changes in actin structures.

    IV.   Invasion and migration assays in response to extracellular matrix components.

Therefore, we have successfully completed a major proportion of the experiments proposed for the first 18 months of study. The data gathered from this initial phase agrees with our hypothesis that Rac3 is important for breast cancer metastasis. *In vitro* analysis of highly metastatic breast cancer cell lines expressing dominant negative Rac3 demonstrated actin cytoskeletal projections, invasion, and migration in response to extracellular matrix components.

We have also initiated the proposed task 2 for in vivo analysis of the effect of creating tumors expressing rac3 mutants in a nude mouse model. As proposed, we are currently in the process of designing and adapting a fluorescence confocal microscope to specifically image fluorescent protein (FP)-tagged cells inside live mouse tumors. Moreover, we have designed a light box to visualize the gross morphology of FP-tagged tumors in live mice.

Thus, the proposed research is progressing ahead of schedule and we are confident of completing the study in three years and making a significant contribution to understanding the role of Rac3 and PAK in breast cancer metastasis.

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